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Comparative *in-vitro* Intrinsic Clearance of Imipramine in Multiple Species Liver Microsomes: Human, Rat, Mouse and Dog

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Abstract

Imipramine is a renowned tricyclic anti-depressant molecule. It is known to be highly potent and efficient as compared to any other antidepressants available. There are numerous literature reports which elucidate the metabolic pathways of Imipramine. There are also various reports showing the effect of substrate and time on metabolism of Imipramine. Most of the analytical methods used in these studies were HPLC and or LC-MS. These studies have also been extended to identification of specific isoforms of cytochrome P450 family responsible for the metabolism of Imipramine as is outlined. Further, LC-MS based studies have also been reported on the metabolism of Imipramine in *in-vitro* systems including human liver microsomes and its extrapolation on human oral bioavailability data. However, till date, there has been no published report on the systematic metabolic stability of this molecule in pooled mouse and dog liver microsomes. The present study was undertaken with the aim to understand the metabolic stability of imipramine in liver microsomes of different species including human, wistar rat, CD1 mouse and male beagle dog. We have also attempted to outline the differences between the metabolic stability in these four species. The reaction samples were scanned for specific m/z values corresponding to reported metabolites, which in turn confirms microsomal metabolism.

Keywords: Metabolic stability; Human Liver Microsomes (HLM); Rat Liver Microsomes (RLM); Mouse Liver Microsomes (MLM); Dog Liver Microsomes (DLM)

Introduction

Imipramine, a prototype of the tricyclic antidepressant class, is one of the most effective molecules available for the treatment of depression. Its main mechanism of action is inhibition of biogenic amine transporters. Because of its unsurpassed effects, it is considered as "Gold standard" for assessing novel antidepressant molecules. Till now, almost all types of *in-vivo* and *in vitro* studies have been undertaken ranging from method development and impurity profiling to *in-vivo* and *in-vitro* studies with help of sophisticated instrumentations like HPLC and LC-MS [1]. In case of *in-vivo* and *in-vitro* studies, researchers have mainly contributed on outlining metabolism of imipramine. In the literature, various phase I and phase II metabolites have been reported [2]. Among the phase I metabolites, the major metabolites are desipramine, 2-hydroxy imipramine, 10-hydroxy imipramine, N-oxide derivative of imipramine. The phase II metabolites mainly include glucuronide conjugates [3]. Metabolism studies were initially started in human liver microsomes, which were then extended to rat liver microsomes [4] and mouse liver microsomes [5]. Numerous reports have elucidated the structures of major metabolites and simultaneously rate of formation of those metabolites in different species [6].

Present study aims to address the metabolic stability of imipramine as determined using the *in-vitro* systems. Since there are no reports about the direct comparison of metabolic stability of Imipramine in various species, the present study was planned to generate the hitherto unreported comparison data of the interspecies variations with regards to the metabolic stability data of imipramine. The present study, thus, evaluates the metabolic stability of Imipramine in different biological systems and establishes the metabolic capacity of various liver microsomes [7,8].

Materials and methods

Chemicals

Imipramine hydrochloride, (3-(10, 11-dihydro-5H-dibenzo [b,f] azepin-5-yl)-N,N-dimethylpropan-1-amine) was purchased from Sigma Aldrich (St. Louis, MO). Pooled human liver microsomes (HLMs), pooled wistar rat liver microsomes (RLMs), pooled CD 1 mouse liver microsomes (MLMs) and pooled male beagle dog liver microsomes (DLMs) were procured from BD Biosciences (Woburn, MA). Phosphate buffer used was of analytical grade. β-Nicotinamide adenine dinucleotide phosphate reduced tetrasodium salt (NADPH) was purchased from Sigma-Aldrich (St. Louis, MO). HPLC grade water was procured from Milli-Q water system (Millipore, USA). All other chemicals used were of analytical grade and proven purity.

Equipments

Analysis was performed on Agilent 1100 system having quaternary pump system, degasser, column oven and diode array detector. Software for controlling the system was EZChrom Elite™. Standard stock solutions were made in Corning 96 well plate. Multichannel pipettes and single channel pipettes were of Eppendorf. Eppendorf Centrifuge 5810R was used for centrifugation of the samples. Metabolites were analyzed on Shimadzu LC-MS 2010 EV comprising of binary pump, degasser, column oven, diode array detector and controlled by LCMS solution (Version 3) (Shimadzu, Japan). HPLC and LC-MS analysis was done on Waters XBridge C18 Column, 5 µm, 4.6 x 250 mm (Waters, MA). Vortex Genie 2 (Scientific Industries, NY, and USA) was used for quenching the reaction after adding chilled Acetonitrile (ACN).

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Experimental

HPLC method development

An HPLC method was developed using ACN and 0.1% Trifluoroacetic acid (TFA) in water with a flow rate of 1 ml/min. Gradient mode was optimized to elute out soluble proteins at early stage and allowing parent molecule as well as its metabolites to separate at a fair resolution. This method was then checked for linearity using HLM as blank protein. Finally optimized method is shown in Table 1. Chromatograms were scanned at 215 nm for presence of imipramine.

Selectivity and specificity

For selectivity and specificity, six different batches of drug-free liver microsomes of different species (without analyte) were analyzed for the exclusion of any endogenous co-eluting interference at the peak region of analyte.

Linearity of method

A protocol was developed and optimized for estimating extraction efficiency of the method using HLM as model protein. As per the protocol, stock solutions of imipramine were prepared from 40 mm to 0.055 mm by serial dilution using DMSO as a diluent. The reaction was then started in microcentrifuge tubes with 485.75 µl of pre-warmed phosphate buffer and mixing it with 1.25 µl of respective stock solutions individually (so as to maintain 0.25% DMSO as final concentration in the assay). The solution was warmed at 37°C for 5 min and then 13 µl of liver Microsomes (0.5 mg/ml protein concentration) was added. Reaction was quenched by addition of 300 µl of chilled ACN and vortexed to precipitate the proteins. The solution was then centrifuged at 9000 rpm for 15 min to sediment the precipitates. Supernatant was taken in HPLC vials and analysis was performed using 20 µl as injection volume. The analysis was done in triplicate.

Areas of the peaks were plotted against the respective concentrations and linearity of the method was established as per the data shown in Table 2. The linearity is also shown graphically in Figure 1.

Precision and accuracy

The precision and accuracy of the method were assessed by performing replicate analyses of samples at four concentration levels (100 µm, 11.11 µm, 1.23 µm and 0.13 µm). The inter-day (n = 5) and intra-day (n = 3) precisions were determined at three levels of QC samples and expressed as Relative Standard Deviation (R.S.D). The accuracy was calculated as percent difference in the observed and nominal concentrations of above mentioned samples.

Time (min)	0.1% TFA in water	ACN	Mode
1.01→ 7.0	70 → 10	30 → 90	Linear gradient
7.0 → 10.0	10 → 0	90 → 100	Linear gradient
10.0 → 13.0	0	100	Isocratic
13.0 → 13.01	0 → 70	100 → 30	Sharp gradient
13.01 → 17.00	70	30	Reequilibrium

Table 1: Optimized HPLC method for assessing imipramine in *in vitro* samples.

Concentration (µM)	Area (Mean ± S.D.)	Regression equation	R^2 value
100	12519123 ± 0.45	**y = 12523x + 5620**	**1**
33.33	4209220 ± 0.03		
11.11	1403757 ± 0.06		
3.7	463231 ± 0.08		
1.23	149637 ± 0.29		
0.41	52186 ± 0.13		
0.137	17348 ± 1.38		

Table 2: Calibration curve (n=3), Average of all area is taken into consideration.

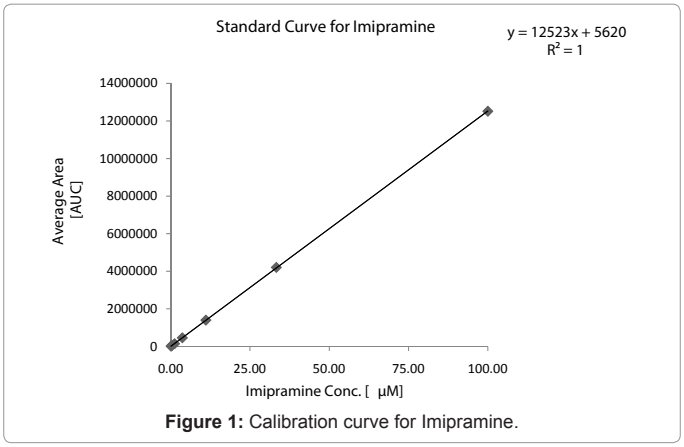


Figure 1: Calibration curve for Imipramine.

Mass parameter	Value
Acquisition Mode	Scan/SIM
Event time	1 Sec
Detector voltage	1.5 Kv
Interface	ESI
Polarity	Positive
Interface Temperature	250 °C
CDL Temperature	250 °C
Nebulizing gas flow	1.5 L/min
Heat block	200 °C
Acquisition time	500 mSec

Table 3: Optimized Mass parameters.

Nominal Conc. (µM)	Experimental Conc. (µM) (Mean ± S.D.)	Precision %	Accuracy %
100	99.32 ± 1.59	1.60	99.32
11.11	11.93 ± 0.30	2.48	107.37
1.23	1.30 ± 0.08	6.11	105.72
0.13	0.13 ± 0.01	10.01	99.27

Intra-day Data (n=3)

Nominal Conc. (µM)	Experimental Conc. (µM) (Mean ± S.D.)	Precision %	Accuracy %
100	99.30 ± 1.26	1.27	99.30
11.11	11.72 ± 0.35	3.01	105.51
1.23	1.22 ± 0.12	9.69	99.53
0.13	0.12 ± 0.01	9.71	94.93

Inter-day Data (n=5)

Table 4: Precision and accuracy data for Imipramine samples.

Recovery and matrix effect:

For the determination of matrix effect and recovery, first blank matrix was spiked with known concentration of imipramine at four concentration levels and analyzed using the developed method. The standard solutions of imipramine of same concentrations were then prepared in the diluent, and analyzed. The peak areas of the extracted samples and the standard solutions were obtained and recovery was calculated using the following equation:

% recovery = (peak area of extracted sample*100) / peak area of standard solution

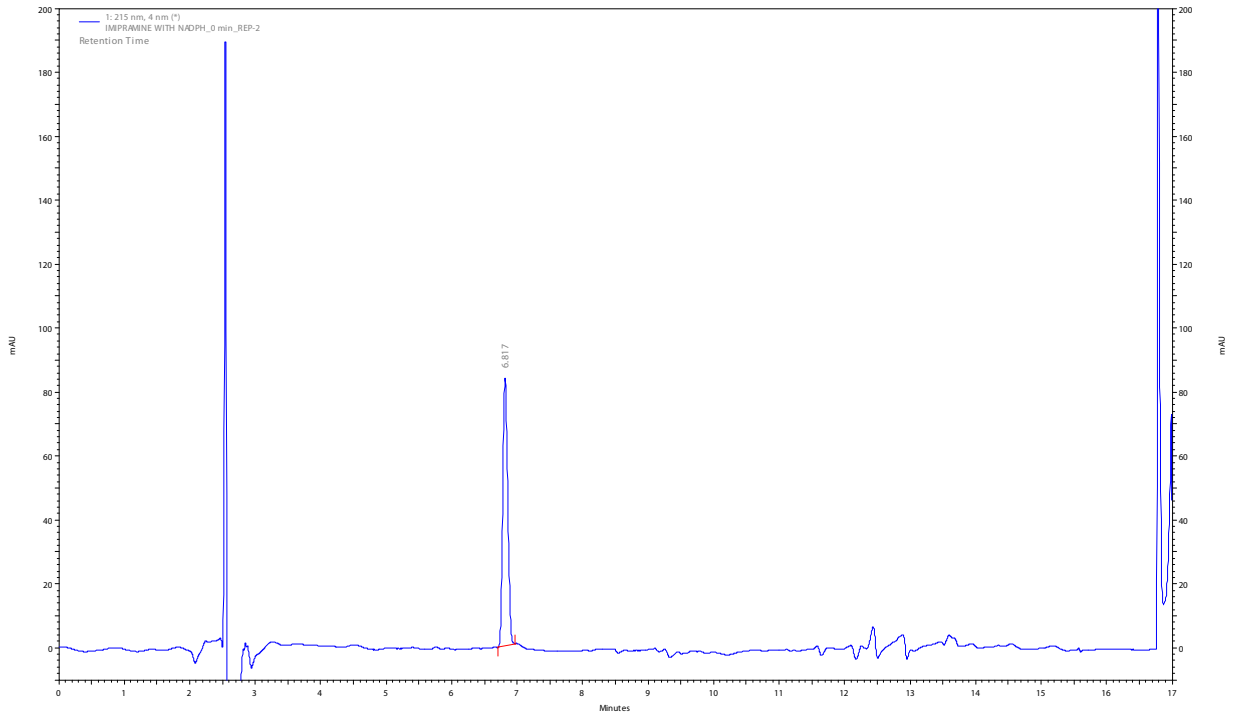


Figure 2: Representative HPLC chromatogram of imipramine.

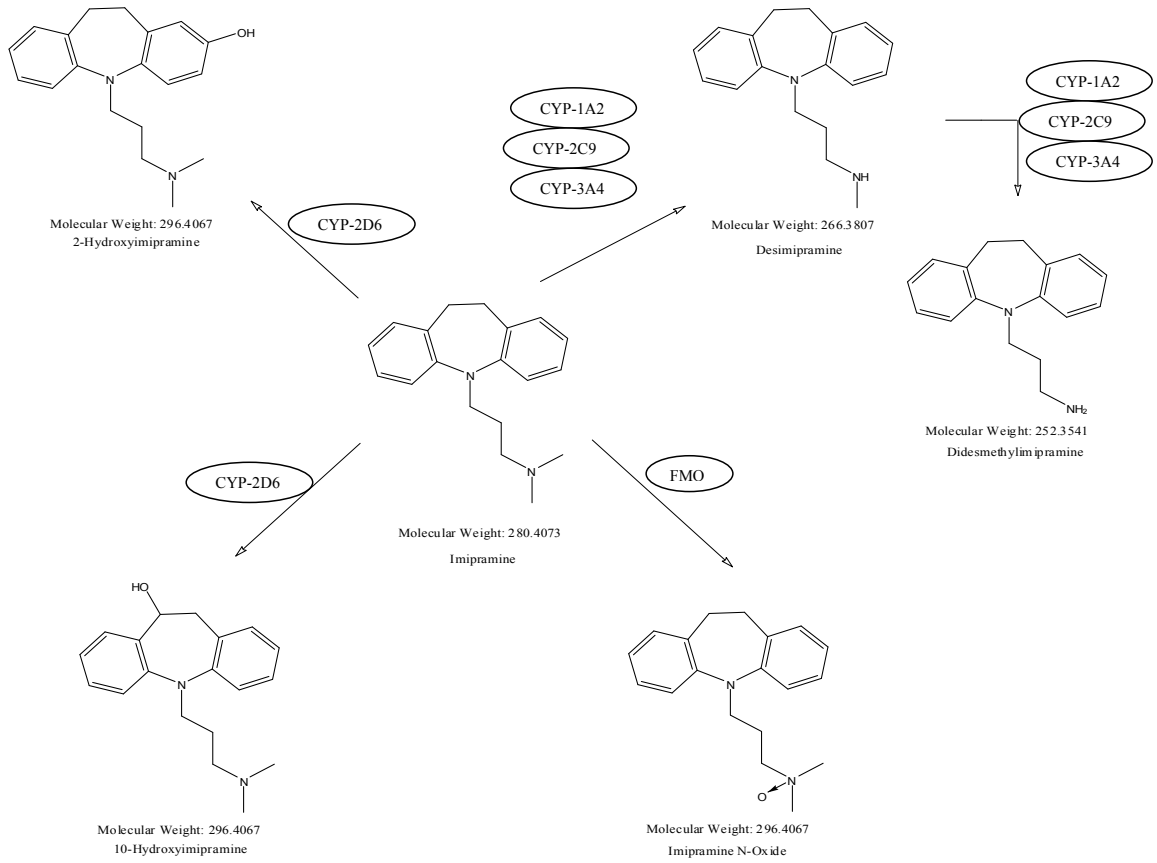


Figure 3: Metabolite generation from parent molecule of Imipramine with respective enzymes. (FMO-Flavin containing Monooxygenases).

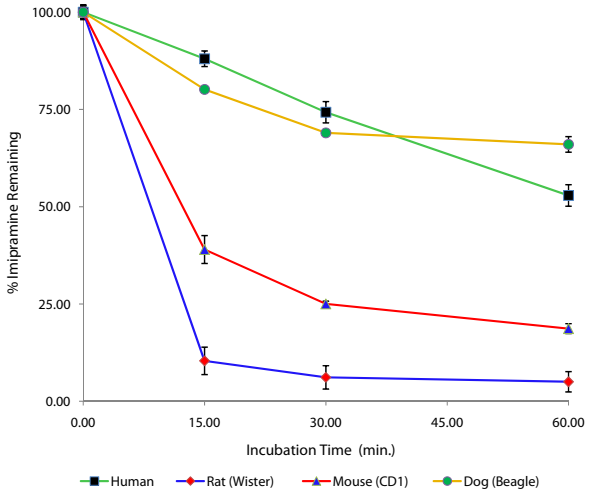


Figure 4: % Imipramine remaining in different species at different time intervals.

Moreover, the matrix effect was determined (n=6) by comparing response of neat solutions and processed samples at 0.13 µm level. The extraction efficiency and the recovery are discussed under the results.

In vitro studies

Same protocol was followed for *in-vitro* metabolic study. For reaction samples, 50 µl of 10 mM NADPH was added with 435.75 µl phosphate buffer and for the control samples NADPH was replaced with 50 µl of phosphate buffer in control samples. The microcentrifuge tubes were kept in shaking water bath at 300 rpm. At the pre-defined time points (i.e. at 0 min, 15 min, 30 min and 60 min), the samples were withdrawn and quenched by addition of ACN followed by vigorous vortexing [9]. Same procedure was followed for control samples and analysis was done as per method mentioned earlier.

Area of imipramine was plotted against time points to assess amount of drug remaining in bioanalytical samples generated. All the samples thus generated were preserved for 60 min (-20°C) and were utilized for LC-MS study to identify and confirm the reported metabolites. Based on the presence of parent compound, intrinsic clearance was calculated using the following equation:

Intrinsic clearance CL_{int} = 0.693 / (*In vitro* $t_{1/2}$ * microsomal protein concentration)

(µl*min^{-1}*mg^{-1})

Metabolite Identification

All the samples generated during the *in-vitro* study were directly utilized for LC-MS study in order to identify the metabolites reported in literature. The known mass fragments of metabolites were given for Selective Ion Monitoring (SIM) mode, i.e. m/z of 280 for imipramine, 266 for desimipramine, 252 for didesmethylimipramine, and 296 for 2-and10-hydroxyl imipramine as well as for imipramine N-Oxide. Optimized mass parameters are reported in Table 3.

Results

HPLC method development

Initially method was started with high amount of aqueous phase in order to elute matrix components and then gradient phase was given to elude the compound of interest as well as its metabolites. A representative chromatogram is Figure 2.

Linearity of the method

After establishing elution pattern of the drug, different concentrations of imipramine were injected and analyzed on HPLC system and integrated accordingly. Then the area was plotted against final effective concentration range of 100 µm to 0.138 µm. Regression equation was calculated on basis of graph and is tabulated Table 2. Lower limit of quantification (LLOQ) of imipramine was found to be 0.1 µm for this method.

Accuracy and precision

Selected concentrations were back calculated using the calibration curve equation and result is represented in Table 4. All the samples showed <15% variability which allows them to fall in acceptance criteria.

Recovery and matrix effect

The experimental recovery was found to be more than 95%. Additionally, the results also indicated that there was no matrix effect, meaning, no interference on the quantification of analytes from the matrix, even at LLOQ Figure 3.

In vitro studies

All the samples generated during metabolic stability study were analyzed as per aforementioned method. Peak area at 0 min sample was considered as 100% of imipramine present in the sample. Reduction in area of peak of interest was observed with subsequent time points. Based on area values, % drug remaining at each time point was calculated and plotted graphically. Representation of the data is made in Figure 4. It is evident from the Figure 4 that the rate of metabolism of imipramine is different in all the species under consideration. Maximum metabolism was observed in wistar rat (RLM) which was followed by CD1 mouse

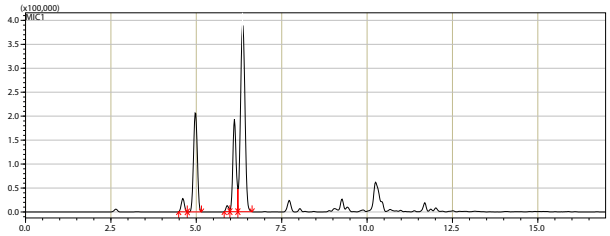
Species	Time points (min)	Average % drug remaining (Mean ± S.D.)	Average % drug metabolized (Mean ± S.D.)
Human	0	100	0
	15	88.03±2.0	11.97±4.65
	30	74.28 ± 2.74	25.72 ± 2.06
	60	52.90 ± 2.76	47.10 ± 3.56
Rat (Wistar)	0	100	0
	15	10.38 ± 3.53	89.62 ± 3.52
	30	6.15 ± 2.99	93.85 ± 0.79
	60	5.01 ± 2.61	94.99 ± 0.14
Mouse (CD 1)	0	100	0
	15	39.00 ± 3.59	61.00 ± 4.08
	30	25.02 ± 0.72	74.98 ± 3.13
	60	18.68 ± 1.27	81.32 ± 0.33
Dog (Beagle)	0	100	0
	15	80.15± 0.23	19.85 ±1.26
	30	68.99 ±0.01	31.01±0.61
	60	66.02 ± 2.0	33.98 ± 0.80

Table 5: Metabolic stability data of Imipramine in different species.

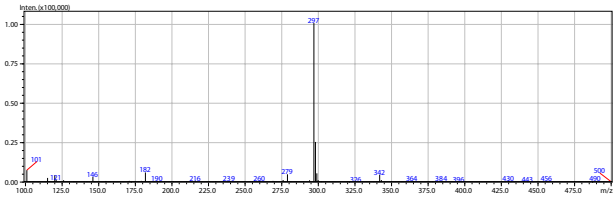
No.	Species	Half Life (min) (Mean ± S.D.)	Intrinsic Clearance (µl*min^{-1}*mg^{-1}) (Mean ± S.D.)
1	Human	65.31 ± 2.0	21.22 ± 0.7
2	Rat	4.589 ± 0.6	302.00 ± 3.1
3	Mouse	11.04 ± 2.5	125.53 ± 2.1
4	Dog	46.98 ± 3.6	29.49 ± 2.2

Table 6: Half life and intrinsic clearance data for Imipramine in all the species.

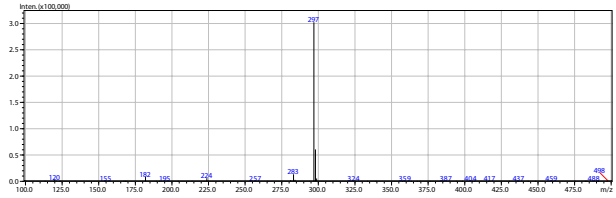
(a) Ion chromatogram of metabolites of Imipramine in MLM.



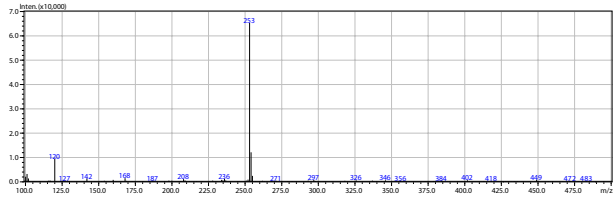
(b) Mass chromatogram at Rt-4.606 min for base peak of 297 m/z



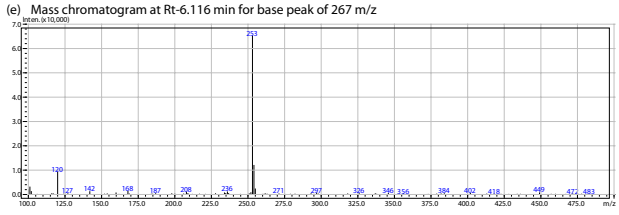
(c) Mass chromatogram at Rt-4.974 min for base peak of 297 m/z



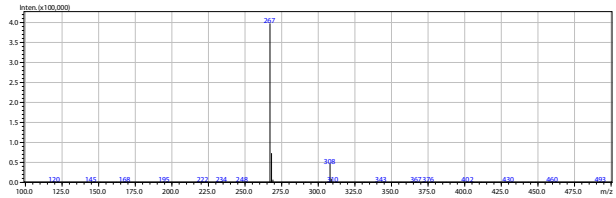
(d) Mass chromatogram at Rt-5.905 min for base peak of 253 m/z



(e) Mass chromatogram at Rt-6.116 min for base peak of 267 m/z



(e) Mass chromatogram at Rt-6.116 min for base peak of 267 m/z



(f) Mass chromatogram at Rt-6.358 min for base peak of 297 m/z

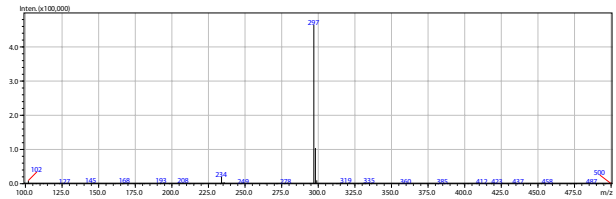


Figure 5: Extracted Ion chromatogram for SIM mode and their respective mass chromatograms at specified retention time values.

Type of Microsomes	Age & Sex of the Animals	Enzymes Present	Specific activity P-450 (pmole /mg)
Pooled Human Liver Microsomes (HLM)	Pool of liver from 24 (Male and Female) donors 24-77 years of Age	CYP1A2	300
		CYP3A4	
		CYP2C9	
		CYP2D6	
Pooled Rat Liver Microsomes Wister Han (RLM)	Pool of liver from 17 male rats, 8-10 weeks of Age	CYP1A2	820
		CYP3A4	
		CYP2C9	
		CYP2D6	
Pooled Mouse Liver Microsomes CD1 (MLM)	Pool of liver from 8 male mice, 11 weeks of Age	CYP1A2	740
		CYP3A4	
		CYP2E1	
		CYP4A11	
Pooled Dog Liver Microsomes Beagle (DLM)	Pool of liver from 4 male dogs, aged ≥ 12 months	CYP1A2	260
		CYP3A4	
		CYP2C9	
		CYP4A11	

(Information provided by BD Gentest™ , Woburn, MA 01801)

Table 7: Details of Microsomes and CYP-450 enzyme specific activity.

(MLM) human (HLM) and then in dog (DLM). Rate of formation of metabolites was also found be varying depending upon species selection. The result metabolic stability of imipramine was thus established in all four species, which were found to be experimentally 52.90 ± 2.76%, 5.01 ± 2.61% , 18.68 ± 1.27% and 66.02 ± 2.0 of drug remaining in HLM, RLM, MLM and DLM respectively Table 5. Intrinsic clearance and half life were calculated and are reported in Table 6.

Metabolite Identification

Samples were scanned under the optimized conditions mentioned earlier. SIM mode was used to selectively scan the ions of interest. Based on ion chromatograms, all the metabolites were confirmed in *in-vitro* samples. Representative chromatogram is shown in Figure 5 which indicates the presence of the metabolites formed during this *in-vitro* exposure.

Conclusion

Till date almost all metabolic pathways of imipramine have been established with proper framework of data. Typically, all the phase I and II metabolites have also been reported from *in-vivo* as well as *in vitro* metabolism studies. The effects of substrate concentration and time profile of metabolite generation has also been reported earlier. However, the metabolic stability across the species and the species differences was the topic unaddressed till date. The, present study has projected the outline of the same objective and as a result, metabolic stability in different species was quantified with HPLC and data is represented in the form of $T_{1/2}$ and intrinsic clearance in all four species in Table 6. The imipramine is metabolized in pooled Human, Rat, Mouse and Dog liver microsomes by different cytochrome P450 isoforms including CYP1A2, 2D6, 2C9, 2C19 and 3A4 to a range of metabolites. The microsomes preparations of all four species have different specific activity of cytochrome P450 enzymes represented in Table 7. The Rat and Mouse liver microsomes showed similar metabolic pattern of imipramine in form of intrinsic clearance as they contain high specific activity of cytochrome P450 enzymes. In Human and Dog low intrinsic clearance values were reported due to long half life of 65.31 and 46.98 min respectively. The present data would be helpful for clinical research, if imipramine is taken up as a standard for checking the metabolic stability of any NCE.

Over and above, the present study also reports for the first time the metabolic stability of imipramine in CD1 MLM and male Beagle Dog DLM. Importance of developed method lies in its universal application in quantifying versatile matrices and the present study encompasses the evaluation of imipramine as well as all its known metabolites in all the four pooled liver microsomes using a single and simple protocol. This data projects imipramine as one of the standards for assessing activity of liver microsomes and the methodologies can be extended for assessing the metabolic stability of NCEs.

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